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Protection against glucose-induced neuronal death by NAAG and GCP II inhibition is regulated by mGluR3

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Abstract

Glutamate carboxypeptidase II (GCP II) inhibition has previously been shown to be protective against long-term neuropathy in diabetic animals. In the current study, we have determined that the GCP II inhibitor 2-(phosphonomethyl) pentanedioic acid (2-PMPA) is protective against glucose-induced programmed cell death (PCD) and neurite degeneration in dorsal root ganglion (DRG) neurons in a cell culture model of diabetic neuropathy. In this model, inhibition of caspase activation is mediated through the group II metabotropic glutamate receptor, mGluR3. 2-PMPA neuroprotection is completely reversed by the mGluR3 antagonist (S)-α-ethylglutamic acid (EGLU). In contrast, group I and III mGluR inhibitors have no effect on 2-PMPA neuroprotection. Furthermore, we show that two mGluR3 agonists, the direct agonist (2*R*,4*R*)-4-aminopyrrolidine-2, 4-dicarboxylate (APDC) and *N*-acetyl-aspartyl-glutamate (NAAG) provide protection to neurons exposed to high glucose conditions, consistent with the concept that 2-PMPA neuroprotection is mediated by increased NAAG activity. Inhibition of GCP II or mGluR3 may represent a novel mechanism to treat neuronal degeneration under high-glucose conditions.

Keywords: apoptosis, diabetes, dorsal root ganglia, glutamate carboxypeptidase II, metabotropic glutamate receptor, *N*-acetyl-aspartyl-glutamate.

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Diabetic neuropathy is a common complication of diabetes, and occurs in up to 60% of diabetic patients. Despite the significant pathology associated with nerve degeneration, the etiology of neuropathy is still unclear. Many pathways may be involved in diabetic neuropathy, such as oxidative stress (Cameron *et al.* 1993; Russell *et al.* 2002; Vincent *et al.* 2002), altered polyol metabolism (Cameron *et al.* 1997), mitochondrial dysfunction (Montal 1998; Russell *et al.* 2002; Huang *et al.* 2003), activation of certain cysteine proteases (caspases) (Russell *et al.* 1999, 2002; Srinivasan *et al.* 2000; Cheng and Zochodne 2003; Schmeichel *et al.* 2003), and regulation of growth factors and their intermediate signaling pathways (Tomlinson *et al.* 1996; Sayers *et al.* 2003). The glutaminergic system has never been investigated as a contributing factor to diabetic polyneuropathy, although there is considerable evidence that glutamate plays an important role in cellular injury and death in several neurodegenerative diseases (Arias *et al.* 1998; Beal 1998; Atlante *et al.* 2001).

There is increasing evidence that the metabotropic glutamate receptors (mGluRs) modulate cellular injury (Vincent and Maiese 2000; De Blasi *et al.* 2001), although

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Abbreviations used: AIDA, (*RS*)-1-aminoindan-1,5-dicarboxylic acid; AMPA, amino-3-hydroxy-5-methylisoxazole-4-propionic acid; caspase, cysteine-requiring aspartate protease; DRG, dorsal root ganglion; DMEM, Dulbecco's modified Eagles medium; EGLU, (*S*)-α-ethylglutamic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCP, II, glutamate carboxypeptidase II; MCPG, (+/–)-α-methyl-4-carboxyphenylglycine; mGluR, metabotropic glutamate receptor; MSOP, α-methylserine-*O*-phosphate; NAAG, *N*-acetyl aspartyl glutamate; NAALADase, *N*-acetylated alpha-linked acidic-dipeptidase; PCD, programmed cell death; 2-PMPA, 2-(phosphonomethyl)pentanedioic acid; SC, Schwann cell.

the role of the mGluRs in diabetic neuropathy is presently unknown. The mGluRs are a subfamily of glutamate receptors that are G-protein-coupled and linked to second messenger systems (Cartmell and Schoepp 2000; De Blasi *et al.* 2001). The eight subtypes are further divided into three groups based on sequence similarity and second messenger system coupling. Group I is coupled to phosphoinositide turnover, groups II and III to adenylate cyclase activity (Tanabe *et al.* 1992). mGluR3, as a member of group II, is negatively coupled to adenylate cyclase, and activation results in decreased cAMP formation, which may have a regulatory effect on apoptosis in DRG neurons (Goswami *et al.* 2000).

GCP II, previously called NAALADase (N-acetylated alpha-linked acidic-dipeptidase), is a neuropeptidase expressed primarily in glial cells that hydrolyzes the neuropeptide *N*-acetyl-aspartyl-glutamate (NAAG) into glutamate and *N*-acetyl aspartate (NAA) (Slusher *et al.* 1990). NAAG is both an agonist at group II mGluRs (Wroblewska *et al.* 1997) and a mixed agonist/antagonist at the *N*-methyl-D-aspartate (NMDA) receptor (Valivullah *et al.* 1994), and is released from neurons at depolarization in a calcium-dependent manner (Tsai *et al.* 1990). The selective GCP II inhibitor 2-(phosphonomethyl)pentanedioic acid (2-PMPA) has been shown to have neuroprotective effects *in vitro* and *in vivo* (Slusher *et al.* 1999; Thomas *et al.* 2001) both by decreasing glutamate formation and by increasing NAAG concentrations (Slusher *et al.* 1999; Cai *et al.* 2002).

This study examines the role of GCP II inhibition in preventing programmed cell death (PCD) in neurons exposed to high glucose. In this model, 2-PMPA, a potent and selective GCP II inhibitor, blocked PCD and promoted neurite growth in a dose-dependent manner. We also show that this neuroprotection is mediated through the mGluR3 receptor, most likely by increasing the extracellular NAAG concentration.

Methods

Materials

Sprague-Dawley rats: Harlan-Sprague Dawley (Indianapolis, IN, USA). ACLAR plastic, Biomedia Gel/Mount: Electron Microscopy Services (EMS), Ft. Washington, PA, USA. Trypsin, B-27, neurobasal medium, Dulbecco's modified Eagles medium (DMEM), Ham's F-12 Mix: Gibco-BRL, Gaithersberg, MD, USA. Nerve growth factor (NGF): Harlan Bioproducts for Science. Pen/Strep/Neo, l-glutamine, selenium, hydrocortisone, β-estradiol, transferrin, bisbenzamide, 5-fluoro-2-deoxyuridine (FUDR), *N*-acetyl-aspartyl-glutamate (NAAG), adeno cAMP, forskolin, bovine pituitary extract, fetal bovine serum (FBS): Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal cleaved caspase-3 antibody: Pharmingen (San Diego, CA, USA). Alexafluor 596 goat anti-rabbit antibody: Molecular Probes (Eugene, OR, USA). Caspatag Caspase-9 Cell Death Detection Kit: Serologicals (Norcross, GA, USA). Apoptag In Situ Detection Kit, Intergen (Purchase, NY, USA). Protein assay reagents: Bio-Rad (South San Francisco, CA, USA). Chemiluminescence reagents: Amersham (Piscataway, NJ, USA). Nitrocellulose: Schleicher and Schuell (Keene, NH, USA). Anti-caspase-9 antibody: Cell Signaling (Beverly, MA, USA). Anti-mGluR2/3 antibody: Upstate Biotechnology (Lake Placid, NY, USA). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies: Santa Cruz (Santa Cruz, CA, USA). Anti-GAPDH antibody: Chemicon (Temecula, CA, USA). 2- (Phosphonomethyl)pentanedioic acid (2-PMPA) and 2-[phosphonomethyl] succinic acid (2-PMSA) provided by Guilford Pharmaceuticals (Baltimore, MD, USA). The mGluR inhibitors group I receptor antagonist (*RS*)-1-aminondan-1,5-dicarboxylic acid (AIDA), group II antagonist (*S*)-α-ethylglutamic acid (EGLU), the group III antagonist α-methylserine-*O*-phosphate (MSOP), and the group II agonist (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate (APDC): Tocris Cookson Inc (Ellisville, MO, USA).

Cell culture

DRG-Schwann cell (DRG-SC) co-culture

DRG-SC co-cultures were prepared as previously described with the exception that SC were not removed from the culture (Russell *et al.* 1999, 2002; Vincent *et al.* 2002). All DRG were grown with endogenous SC, except where otherwise noted, because SC are necessary for the neuroprotective effects of GCP II inhibition and mGluR3 activation (Bruno *et al.* 1997; Slusher *et al.* 1999; Moldrich *et al.* 2001; Thomas *et al.* 2001). Briefly, DRG from 15-day-old embryonic (E15) Sprague-Dawley rats were aseptically plated on air-dried, collagen-coated plastic ACLAR dishes or collagen coated glass cover slips. Dissociated DRG were prepared by incubating whole DRG containing SC in 0.25% trypsin for 40 min at 37°C, centrifuging at 800 × *g* for 5 min, and then removing trypsin. Explant cultures were grown by placing whole DRG containing SC onto collagen-coated prehydrated aclar dishes. Co-cultures were initially grown for 4–5 days (dissociated) or 1 day (explant) in neurobasal medium containing 25 mM glucose (optimal basal glucose for neurons) (Russell and Feldman 1999; Russell *et al.* 1999; Srinivasan *et al.* 2000), with 0.5% B27, 10 ng/mL nerve growth factor, 0.5% Pen/Strep/Neo, and 1.4 mM l-glutamine. Before experimental conditioned media was added, cells were rinsed three times with low phosphate DMEM, and then defined, serum-free medium was added. Defined medium contains DMEM supplemented with 1.4 mM l-glutamine, 30 nM selenium, 10 nM hydrocortisone, 10 nM β-estradiol, and 10 μg/mL transferrin. For all experiments containing 2-PMPA, media containing low phosphate was used as physiological concentrations of phosphate may inhibit GCP II activity (Thomas *et al.* 2000). It was previously determined that low-phosphate medium does not induce neuronal death (data not shown).

Pure DRG culture

Pure DRG cultures were prepared in the same manner as DRG-SC co-cultures, however, initial plating media contained 40 μM FUDR, and FUDR treatment was maintained until the cultures were depleted of SC and fibroblasts.

Pure differentiated SC cultures

SC were collected from the sciatic nerve of day 3 postnatal Sprague-Dawley rat pups, as previously described (Brockes *et al.* 1980;

Delaney *et al.* 1999). Pure SC cultures were maintained in growth medium containing DMEM, 10% FBS, 2 μM forskolin, and 20 μg/mL bovine pituitary extract. Upon 80% confluency SC were differentiated in DMEM with 10% FBS and 100 μM adeno cAMP. After 24 h incubation, cells were rinsed in DMEM, and conditioned medium added in serum-free Schwann cell differentiation medium (SCDM), containing 50% Ham's F-12 Mix, 50% low-glucose DMEM, and 1 × B27 supplement.

Immunohistochemistry

Caspase-3 cleavage

Cultures were fixed after the experimental time point for 30 min with 4% paraformaldehyde in phosphate-buffered saline (PBS), and then stained for active caspase-3 as previously described (Russell *et al.* 1999; Russell *et al.* 2002; Vincent *et al.* 2002). Cultures were incubated in 0.5 μg/mL rabbit polyclonal cleaved caspase-3 antibody for 2 h at 37°C. Cells were then incubated in 5 μg/mL Alexafluor 596 goat anti-rabbit antibody at room temperature for 1 h. Specificity and concentration of the anti-active antibody was determined by immunoadsorption against cleaved peptide. Samples were then co-stained with 1 μg/mL bisbenzamide in PBS for 5 min at room temperature. The bisbenzamide permits DRG and SC nuclei to be easily and separately identified. Cells were finally mounted on slides with Biomedia Gel/Mount, and analyzed at 40× magnification on a Zeiss Axiophot microscope by a blinded observer in a random fashion. For each slide, 10 random fields of 25–100 cells per field were counted, and the percent caspase-3 positive neurons were determined. The percentages for each field were averaged for each condition. Each experiment was repeated a minimum of three times.

Caspase-9 cleavage

Caspase-9 cleavage was assayed in DRG-SC co-cultures using a Caspatag caspase-9 Cell Death Detection Kit. The protocol was followed for adherent cells as previously described (Russell *et al.* 2002). Briefly, conditioned media was added for 2 h alone, and then caspase-9 antibody was added to conditioned media for 1 h. Cells were counterstained with bisbenzamide, and then fixed in 4% paraformaldehyde for 30 min. Cells were analyzed in the same manner as anti-caspase-3 stained cells.

Neurite outgrowth

Cultured DRG explant cultures were grown for 1 day with endogenous SC, and then exposed to experimental conditioned media. Measurements were obtained every 24 h on a Leitz microscope at 5 or 10× magnification, as previously described (Russell *et al.* 1994) by a trained observer blinded to the experimental condition. Briefly, the longest neurite from each of 24 explants per condition was measured each day for the specified number of days, with baseline growth at the start of the experiment being defined as 0 mm. Results were standardized against control conditions. Each experiment was repeated a minimum of 3 times.

TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining

DRG-SC co-cultures were fixed in 4% paraformaldehyde for 20 min and then TUNEL stained using the Apoptag In Situ Detection Kit to detect nuclear fragmentation, as previously described (Delaney *et al.* 2001). Slides were analyzed at 100× on a Zeiss Axiophot microscope by a blinded observer in a random fashion; for each slide, 10 random fields of 25–100 cells each were counted, and the percent TUNEL positive neurons determined for each field. The percentages for each field were averaged for each condition. Each experiment was repeated a minimum of 3 times.

Immunoblot detection of mGluR3 and caspase-9 protein

Western immunoblot was performed as previously described (Delaney *et al.* 2001; Vincent *et al.* 2002). Protein (25 μg per sample) was separated by SDS-PAGE and transferred to nitrocellulose. Primary antibody was diluted 1 : 1000 (antiactive caspase-9) or 1 : 500 (anti-mGluR2/3), followed by secondary horseradish peroxidase-conjugated goat anti-rabbit antibody diluted 1: 2000. After exposure using chemiluminescence reagents, the blot was re-probed for GAPDH at a 1 : 2000 dilution, followed by horseradish peroxidase-conjugated goat anti-mouse antibody at a 1 : 2000 concentration.

Drug treatment

Before beginning experimental drug treatment, DRG-SC co-cultures or pure DRG neuronal cultures were grown for 4–5 days (dissociated cultures) or 1 day (explant cultures) at 37°C. Before addition of drugs, cells were rinsed three times with low phosphate DMEM. All drugs were added simultaneously except in the case of experiments using EGLU and NAAG, in which case any condition with EGLU was subjected to 30-min pretreatment with EGLU. For all other experiments, it was determined that drug pretreatment did not affect the results from receptor blocking or activation. Cells were not kept in conditioned media for longer than 24 h without re-dosing with fresh media.

Statistics

Due to the large number of data points measured, a normal distribution for each of the endpoints was assumed. Overall changes in measures were analyzed using ANOVA, and individual comparisons were made using Student's *t*-test and assuming unequal variances. Levels of significance are as follows: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) and comparisons are made with control, except where otherwise noted. For graphical and descriptive purposes data are presented as mean ± standard error of the mean (SEM). In some cases, results were standardized against controls.

Results

2-PMPA prevents glucose-induced PCD

In order to establish that PCD is induced in a combined system, DRG-SC co-cultures were treated with 20 mM added glucose (45 mM total glucose). The percentage of neurons positive for cleaved caspase-3 and TUNEL staining were increased by high-glucose (Fig. 1). Control neurons show lower cell death than in previous SC free studies (Russell and Feldman 1999; Russell *et al.* 1999), suggesting that the presence of SC reduces PCD.

The GCP II inhibitor 2-PMPA was examined in both control and glucose-treated cells to determine if it could

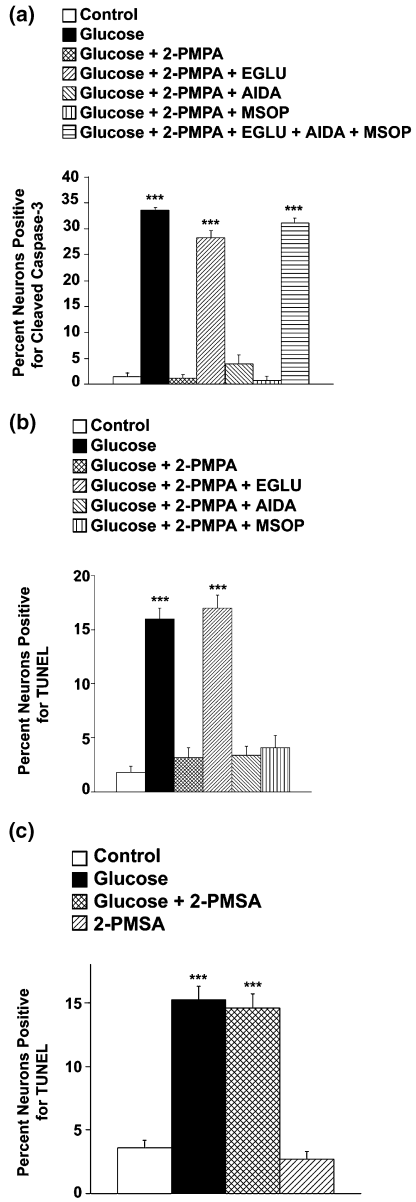

Fig. 1 2-PMPA prevents glucose-induced PCD at 24 h as indicated by the percent neurons positive for cleaved caspase-3 (a) and at 48 h as indicated by TUNEL positive nuclei (b). Cells were treated with 20 mM added glucose ± 6 μM 2-PMPA, or ± 100 μM of one or more mGluR antagonist. The mGluR3 antagonist EGLU was able to reverse the neuroprotective effects of 2-PMPA, both alone and in conjunction with group I and group III antagonists. Neither the group I antagonist AIDA, nor the group III antagonist MSOP was able to reverse 2-PMPA protection. Ten micromoles of the inactive analogue 2-PMSA does not inhibit glucose-induced cell death (c). ***$p < 0.001$.

rescue DRG neurons from glucose-induced PCD. Three separate PCD assays, caspase-3 and -9 cleavage and TUNEL, all showed that 2-PMPA is able to completely rescue DRG neurons from glucose-induced PCD. 2-PMPA is able to completely rescue DRG neurons from caspase-3 cleavage for up to at least 24 h (Fig. 1a), and DNA fragmentation up to at least 48 h (Fig. 1b). It was determined from our own dose-response experiments and published results (Slusher *et al.* 1999) that the optimal concentration of 2-PMPA in culture to prevent PCD is 6 μM. There is increasing toxicity above this optimal dose. SC are in general less susceptible to glucose-induced damage than are DRG neurons (Delaney *et al.* 2001), but are able to prevent neuronal injury, making them an ideal target for GCP II inhibition.

In order to ascertain that 2-PMPA inhibition was specific for GCP II, the inactive analogue 2-PMSA was tested as a potential inhibitor of glucose-induced cell death. As was expected, 2-PMSA had no ability to rescue DRG from PCD within the co-culture system (Fig. 1c). Neither 2-PMPA nor EGLU alone had any effect on caspase-3 cleavage or TUNEL activation (data not shown).

mGluR3 antagonists are able to reverse neuroprotection provided by 2-PMPA

One protective effect of 2-PMPA is hypothesized to be increased action of NAAG at the mGluR3 receptor. We therefore tested whether an mGluR3 antagonist along with 2-PMPA would block 2-PMPA mediated neuroprotection in this system. For comparison, we also used several other mGluR antagonists. We found that the mGluR3 antagonist EGLU was able to reverse protection provided by 2-PMPA in all 3 PCD assays used: caspase-3 and -9 cleavage, and TUNEL (Figs 1 and 2). The addition of the mGluR3 antagonist EGLU reversed the protection provided by 2-PMPA in all instances. However, this reversal of protection was not seen with the group I mGluR antagonist AIDA, nor the group 3 mGluR antagonist MSOP. However, when all three mGluR3 antagonists were combined (AIDA, MSOP, and EGLU), reversal of protection was seen once again (Fig. 1). All antagonists were added at an identical concentration of 100 μM, sufficient to block their specific receptors.

Similar to previous observations in DRG neurons, caspase-9 cleavage occurs early in the PCD cascade with maximal cleavage at 3 h (Russell *et al.* 2002), as shown in Fig. 2(a).

The caspase-9 immunoblot showed similar results to immunohistochemistry (Fig. 2b). In general, immunoblotting is less sensitive than immunohistochemistry in DRG-SC co-cultures, due to the relatively large proportion of SC protein (with considerably less cleaved caspase-9) compared to DRG protein. However, the immunohistochemical results in co-cultures, which permit measurement of PCD in DRG alone, were confirmed with immunoblotting for caspase-9 protein up-regulation and cleavage. The immunoblot shows an increase in both total caspase-9 protein levels as well as an increase in the 37 kDa cleavage product, showing up-regulation of caspase-9 in glucose-treated cells as compared to cells treated with glucose and 2-PMPA combined.

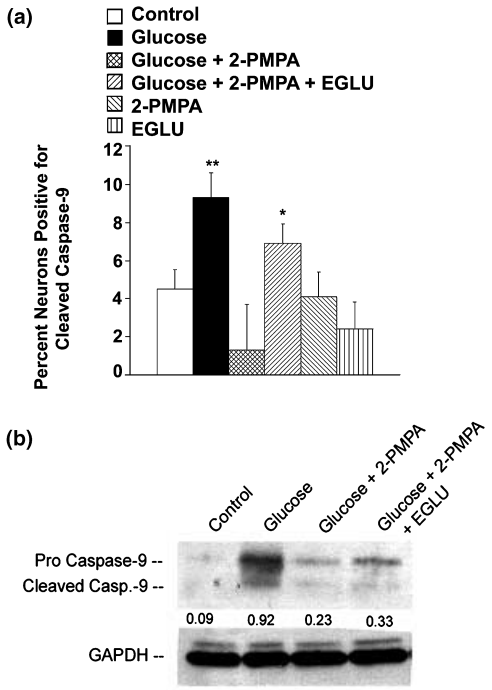

Fig. 2 Immunohistochemistry (a) and immunoblot (b) for caspase-9 activation show an increase in caspase-9 activation at 3 h after addition of extra glucose. In the immunoblot, both the 51 kDa caspase-9 zymogen and the 40 kDa cleavage product are increased with added glucose (25 μg protein loaded per lane) at 3 h. Caspase-9 cleavage is partially reversed by the group II mGluR antagonist EGLU. Cells were treated with 20 mM glucose ± 6 μM 2-PMPA, or ± 100 μM EGLU. The housekeeping protein GAPDH was detected at 35 kDa on the same blot and shows equal protein loading in each lane. The ratio of caspase-9 cleavage product to GAPDH is shown above the GAPDH blot. $^*p < 0.05$.

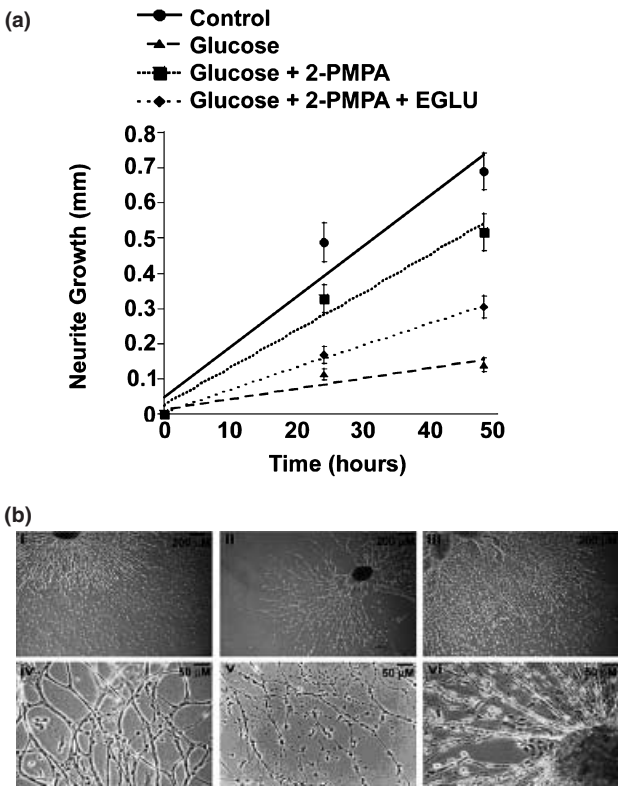

Fig. 3 2-PMPA is able to promote DRG explant neurite growth (DRG-SC co-cultures) in the presence of glucose. Neurite growth in mm (a) and light micrographs showing neurite length and density (b) at low (i–iii) and high (iv–vi) magnifications. Explants plated in the presence of glucose (ii and v) have shorter and sparser neurites, or show evidence of neurite beading and degeneration compared to control explants (i and iv). Six micromolar 2-PMPA promotes growth in the presence of 20 mM glucose (iii and vi). 2-PMPA is able to reverse the effects of glucose on rate of neurite growth, an effect that is partially inhibited by the mGluR3 antagonist EGLU. Neither the group I antagonist AIDA nor the group II antagonist MSOP had any effect on 2-PMPA protection. 2-PMPA alone had no effect on growth (data not shown).

The ratio of caspase-9 cleavage product to GAPDH was ninefold higher with added glucose than control. In contrast, the caspase-9 cleavage product was reduced in the presence of 2-PMPA and increased by addition of EGLU using both immunohistochemistry and immunoblotting. As with added-glucose alone, there was an increase in total caspase-9 levels in the presence of EGLU.

2-PMPA promotes neurite growth in the presence of elevated glucose concentrations

In order to assess the effect of 2-PMPA on glucose-induced axonal degeneration and slowed growth, mean neurite growth of explant DRG-SC co-cultures were measured. In the presence of added glucose, there was an overall reduction in neurite growth (Fig. 3a) at all time points compared to control ($p < 0.001$), as well as degeneration of neurites with continued exposure to glucose (Fig. 3b). In contrast, 2-PMPA maintained normal neurite growth and viability in high glucose conditions. The mGluR3 antagonist EGLU blocked this 2-PMPA mediated effect at all time points ($p < 0.001$). Observed differences continued to 72 h (data not shown). Neither the group I antagonist AIDA, nor the group III antagonist MSOP, were able to reverse 2-PMPA preservation of neurite growth under high glucose conditions. 2-PMPA alone and EGLU alone had no effect on neurite growth compared to control (data not shown).

NAAG is neuroprotective against PCD

Because it is believed that 2-PMPA's neuroprotective effects stem at least in part from NAAG's agonist activity at the mGluR3 receptor (Slusher *et al.* 1990; Wroblewska *et al.* 1993, 1997; Neale *et al.* 2000), we determined if NAAG directly prevented glucose induced PCD. We added 100 μM NAAG to cells treated with added glucose, and measured both caspase-3 cleavage (Fig. 4a) and TUNEL assays

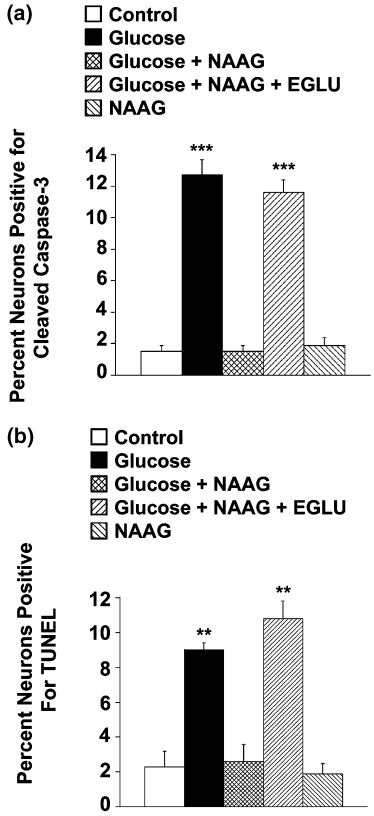

Fig. 4 100 μM NAAG is neuroprotective against glucose-induced cell death as measured by the percentage of neurons positive for cleaved caspase-3 at 24 h (a) and TUNEL at 48 h (b). The mGluR3 antagonist EGLU is able to reverse the effects of NAAG, suggesting that NAAG neuroprotection is mediated through the mGluR3 receptor. NAAG alone has no effect on caspase-3 activation. $^{**}p < 0.05$, $^{***}p < 0.001$.

(Fig. 4b). NAAG reduced glucose-induced caspase-3 and TUNEL activation, and EGLU was able to reverse protection, indicating that NAAG is acting through mGluR3. NAAG alone had no effect on neuron survival.

NAAG promotes neurite growth in the presence of elevated glucose concentrations

In order to ascertain that NAAG promotes neurite growth in the presence of elevated glucose, neurite growth was measured for DRG explants for 72 h (Fig. 5). In the presence of glucose, neurite growth was slowed compared to control at all time points ($p < 0.001$), however, NAAG was able to reverse this trend. The mGluR3 antagonist EGLU blocked NAAG protection ($p < 0.001$ compared to control). Neither NAAG nor EGLU alone had a significant effect on neurite growth as compared to control (data not shown).

The selective mGluR3 agonist APDC is able to prevent glucose-induced PCD

In order to determine that 2-PMPA's and NAAG's neuroprotective effects result from agonist activity at the mGluR3

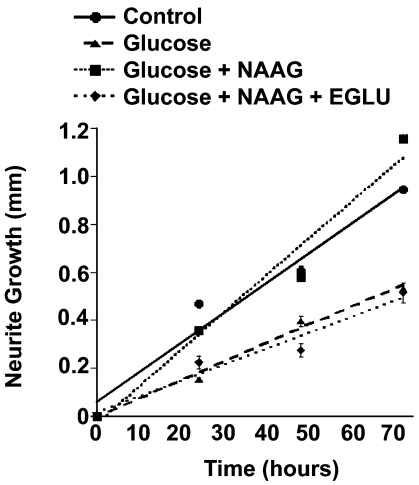

Fig. 5 NAAG is able to promote neurite outgrowth in the presence of 20 mM added glucose over a time period of 72 h. This effect is completely inhibited by the mGluR3 antagonist EGLU, suggesting that NAAG-mediated prevention of slowed axonal growth is mediated through mGluR3. NAAG alone had no effect on growth (data not shown).

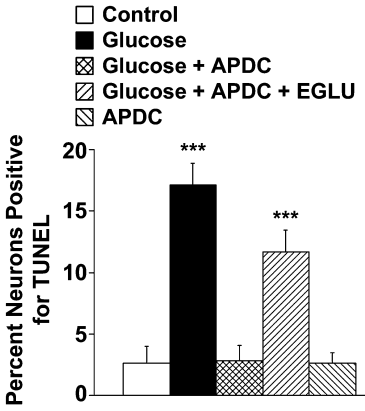

Fig. 6 The selective mGluR3 agonist APDC is neuroprotective against 20 mM added glucose. At a concentration of 100 μM, APDC prevents PCD as measured by the percent neurons positive for TUNEL. The mGluR3 antagonist EGLU is able to reverse protection afforded by APDC. $^{***}p < 0.001$.

receptor, a selective mGluR3 agonist was examined as a potential inhibitor of PCD. We found that APDC was able to prevent glucose-induced PCD, and was reversed by EGLU (Fig. 6). APDC alone had no significant effect. This result demonstrates that a known mGluR3 agonist produces similar effects to NAAG and 2-PMPA.

mGluR3 agonists are not protective in the absence of SC

To determine if SC are necessary for mGluR3 neuroprotection, we tested whether 2-PMPA, NAAG, or APDC provided protection against glucose-induced cell death in DRG

neurons in the absence of SC (Fig. 7). We found that none of the three agents provided protection, indicating that glial-neuronal signaling is crucial for mGluR3-mediated neuroprotection.

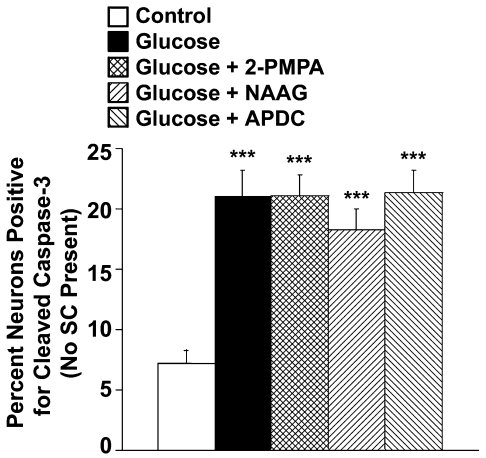

Fig. 7 2-PMPA, NAAG and APDC do not protect DRG neurons grown without SC. Glucose-induced cell death was measured by percent neurons positive for cleaved caspase-3 at 24 h. This suggests that SC are necessary for mGluR3-mediated neuroprotection. ***$p < 0.001$.

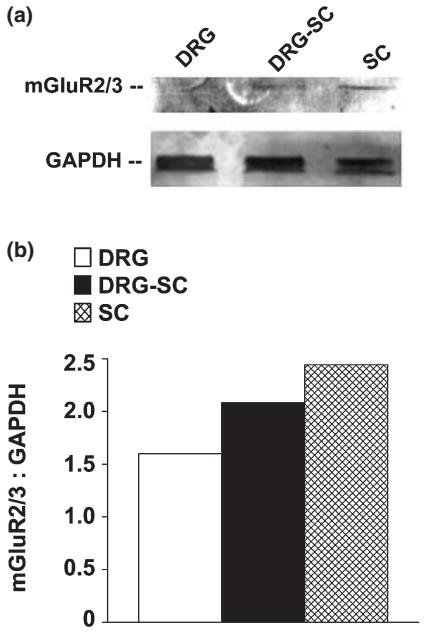

Fig. 8 mGluR3 receptor is localized on SC rather than DRG. Pure DRG neuron cultures show no immunoreactivity to anti-mGluR2/3 antibody, while 100 kDa bands corresponding to mGluR3 are seen in DRG-SC co-cultures and pure SC cultures (a). A histogram showing the ratio of mGluR2/3 : GAPDH housekeeping protein indicates that immunoreactivity for mGluR2/3 is higher in pure SC cultures than in DRG-SC co-cultures (b), implying that the mGluR3 receptor is located on SC rather than on DRG neurons.

mGluR3 is expressed in SC but not DRG neurons

In order to determine if mGluR3 is located on SC or DRG, lysates from DRG-SC co-cultures, as well as pure DRG cultures and pure differentiated SC cultures, were analyzed for mGluR3 receptor. Immunoblotting revealed that mGluR3 protein is present in pure SC cultures, and to a lesser extent in DRG-SC co-cultures, but could not be detected in DRG neurons (Fig. 8). This data implies that the receptor is found on SC rather than DRG neurons.

Discussion

Although the glutaminergic system has been implicated in many neurological disorders, including ischemic brain injury, Parkinson's disease, and Alzheimer's disease (Arias *et al.* 1998; Beal 1998; Atlante *et al.* 2001), its role in the pathogenesis of diabetic neuropathy is uncertain. This study indicates a possible regulatory mechanism through GCP II enzymatic cleavage of NAAG into NAA and glutamate. Inhibition of GCP II cleavage of NAAG by 2-PMPA blocks glucose-induced cell death in DRG neurons, an effect that is likely mediated through NAAG agonist activity at mGluR3.

PCD has been characterized *in vitro* by caspase-3 and -9 cleavage, TUNEL staining, and transmission electron microscopy, and shows evidence consistent with neuronal injury and mitochondrial dysfunction (Russell *et al.* 1999, 2002; Srinivasan *et al.* 2000. Similar changes are observed *in vivo* in acute and chronic streptozotocin diabetic rat DRG (Russell *et al.* 1999; Srinivasan *et al.* 2000; De Giorgi *et al.* 2002; Schmeichel *et al.* 2003). Previous studies both in this laboratory and by other groups indicate that cultured neurons, in contrast to non-neuronal cells, require higher basal concentrations of glucose (Korinkova and Lodin 1976; Bottenstein *et al.* 1989; Russell *et al.* 1999, 2002), and that concentrations of glucose in culture greater or less than 25 mM induce a dose-dependent cleavage of caspase-3 and increase in TUNEL staining (Russell *et al.* 1999, 2002), likely through rapid ATP depletion. This relatively high concentration of basal glucose most likely reflects the increased energy requirements and metabolic rate of DRG compared to non-neuronal cells.

In our current DRG-SC co-culture system, PCD in control conditions was approximately 1–3%, which differs from 7 to 15% in DRG cultures with no endogenous SC (Russell *et al.* 1999). This finding is not surprising, as the glial cells likely have neuroprotective effects on neurons, particularly during development or following toxic injury (Davies 1998; Moldrich *et al.* 2001). Furthermore, we have shown that glial cells are necessary in the neuroprotective mechanisms mediated by inhibition of GCP II and NAAG. Several other groups of investigators have found glia to be necessary for mGluR3 activation to be neuroprotective to neurons (Bruno *et al.* 1997; Slusher *et al.* 1999; Moldrich *et al.* 2001). This is

likely due to the presence of mGluR3 on glia rather than neurons. Similar to previous findings (Azkue *et al.* 2000), it is hypothesized that mGluR3 activation induces the release of neuroprotective factors from the glia, such as NGF (Ciccarelli *et al.* 1999), TGFβ (Bruno *et al.* 1998; Thomas *et al.* 1999a; Aronica *et al.* 2000; D'Onofrio *et al.* 2001), or S-100β (Ciccarelli *et al.* 1999), and indirectly protects neurons from PCD. Thus, a DRG-SC co-culture system was used in these experiments, rather than purified DRG neurons as in our previous studies (Russell *et al.* 1998, 1999, 2002; Vincent *et al.* 2002).

GCP II is inhibited both by 2-PMPA and by cell culture medium containing physiological levels of phosphates (Thomas *et al.* 2000). There is some evidence that diabetic patients may have lower than normal blood phosphate levels (Ravenscroft *et al.* 1999), thus in diabetic patients GCP II is likely to be active and to mediate glucose-induced neuronal PCD. 2-PMPA neuroprotection was completely reversed by the group II antagonist EGLU, while groups I and III antagonists had no effect on protection, indicating that 2-PMPA neuroprotection is mediated via the mGluR3 receptor. This is likely due to increased NAAG concentrations, as 2-PMPA inhibits GCP II hydrolysis of NAAG. In order to determine that 2-PMPA is not preventing glucose-induced PCD via non-selective inhibition of GCP II, an inactive analogue 2-PMSA was used (Thomas *et al.* 2001). 2-PMSA failed to either block GCP II or protect against glucose-mediated PCD, leading to the conclusion that the neuroprotection afforded by 2-PMPA is both selective and specific in this system.

In addition to preventing PCD, 2-PMPA promotes neurite outgrowth in neurons treated with added glucose. DRG neurites treated with increased glucose had a reduced rate of growth compared to controls, neurite density was reduced, and neurites showed evidence of degeneration. These changes were similar to those observed in rat sensory neurons *in vivo* (Russell *et al.* 1999). In contrast, 2-PMPA reversed glucose-induced inhibition of neurite growth. Thus the protective effects of 2-PMPA extend beyond cell survival to cell growth.

Because the link between mGluR3 and 2-PMPA neuroprotection was so profound, we determined if selective mGluR3 agonists were able to provide the same sort of neuroprotection. One such agonist, NAAG, is increased by 2-PMPA inhibition of GCP II (Slusher *et al.* 1999), and furthermore is an endogenous agonist at the mGluR3 receptor (Wroblewska *et al.* 1993, 1997; Neale *et al.* 2000). NAAG has been shown to be neuroprotective in a variety of neurological disorders (Thomas *et al.* 2001; Cai *et al.* 2002), and this study showed it to be protective against glucose-induced PCD, able to completely reverse glucose-induced cell death at every time point tested. In addition, NAAG prevented glucose-induced inhibition of neurite growth in explant cultures. This effect was partially blocked by an mGluR3 antagonist. These findings indicate that NAAG is critical in mediating 2-PMPA neuroprotection in this model of diabetic neuropathy.

Supporting the concept that NAAG neuroprotection is mediated via mGluR3, the selective agonist APDC was able to completely reverse glucose-induced PCD, while the mGluR3 inhibitor EGLU blocked this effect. This finding that mGluR3 agonist activity alone is sufficient to prevent glucose-induced PCD is consistent with the concept that in this high glucose system, NAAG agonist activity at mGluR3 is neuroprotective.

In summary, high-glucose conditions in DRG-SC co-cultures results in increased caspase-3 and -9 cleavage, TUNEL-positive nuclei indicating nuclear degradation, and a decreased neurite growth rate. These effects are blocked in a dose-dependent manner by the GCP II inhibitor, 2-PMPA. Prevention of PCD by 2-PMPA is specific, and is mediated at least in part by the mGluR3 receptor. This is indicated by reversal of protection with the mGluR3 antagonist EGLU, neuroprotection afforded by the mGluR3 agonist APDC, and the neuroprotective effects of NAAG. As 2-PMPA prevents hydrolysis of NAAG into is subcomponents, our findings support the concept that 2-PMPA neuroprotection is mediated by NAAG's agonist activity at the mGluR3 receptor. These results support the hypothesis that hyperglycemia induces GCP II hydrolysis, and have important pharmacologic implications for future therapy of diabetic neuropathy.

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